Clontech Laboratories, Inc.

ProteoTuner™ Guard Systems User Manual

PT5169-1 (PR113800)
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I. Introduction

ProteoTuner technology lets you investigate protein function by directly controlling intracellular levels of a protein of interest. This control is exerted at the protein level—rather than at the level of transcription or translation—allowing you obtain the desired intracellular concentration of your protein of interest in as little as one hour.

The ProteoTuner Guard System is based on a DHFR-derived destabilization domain (DD_G) from *E. coli* that can be expressed as an18 kDa tag on your protein of interest (Iwamoto *et al.*, 2010). In the presence of the small (290 Da), membrane-permeant, stabilizing ligand Guard1, the DD_G-tagged protein of interest is stabilized (protected from proteasomal degradation), allowing it to accumulate inside the cell (Figure 1). Ligand-dependent stabilization occurs very quickly: DD_G fusion proteins have been shown to accumulate to detectable levels just one hour after the addition of Guard1.

The ProteoTuner method is not restricted to protein *stabilization*—it can also be used to *destabilize* the tagged protein: When cells expressing the tagged protein of interest are cultured in medium lacking the stabilizing ligand, the tagged protein undergoes proteasomal degradation (Figure 1). As a result, it is possible to fine-tune the amount of stabilized DD_G-tagged protein in the cell by titrating the amount of Guard1 in the culture medium, and to repeatedly stabilize and destabilize the protein of interest using the same set of cells.

NOTE: For effective protein degradation to occur, DD_G-tagged proteins need to have access to proteasomes within the cell. Regions of the cell that lack proteasomes (e.g., the lumen of the ER) will show reduced Guard1-dependent protein regulation.

The ProteoTuner Guard System was developed to complement the ProteoTuner Shield System, which is based on an FKBP-derived destabilization domain (DD_S) and the stabilizing ligand Shield1 (Banaszynski *et al.*, 2006). Both systems can be used together to independently regulate the amount of two proteins of interest in the same cell. For more information on our Shield1-based ProteoTuner System, see the ProteoTuner Shield Systems User Manual (PT4039-1; available at http://www.clontech.com/manuals).

A variety of ProteoTuner System formats are available. Find out more at www.clontech.com

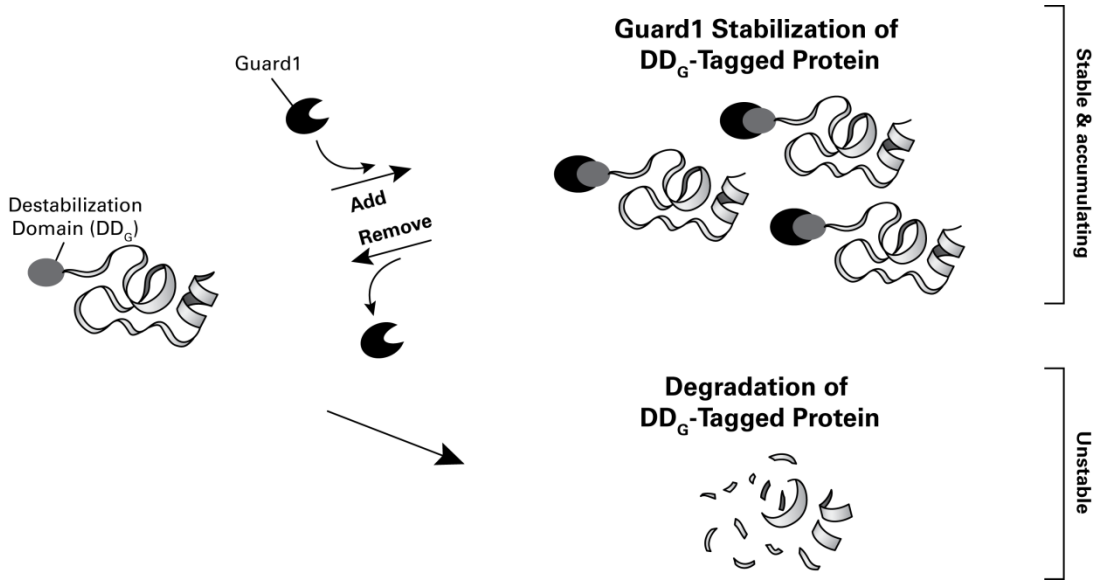

Figure 1. Ligand-dependent, targeted, and reversible protein stabilization. A small, mutated, DHFR destabilization domain (DD_G; grey) is fused to a target protein of interest. The small membrane-permeant ligand Guard1 (black) binds to the DD_G tag, protecting the protein from proteasomal degradation. Removal of Guard1 causes rapid degradation of the entire fusion protein. The default pathway for the ProteoTuner Guard System is degradation of the DD_G-tagged protein, unless Guard1 is present to stabilize it.

II. Additional Materials Required

A. Guard1

Each ProteoTuner Guard System includes 60 µl Guard1 (Cat. No. 635051). Additional Guard1 can also be purchased separately:

- 60 µl Guard1 (Cat. No. 635051)
- 500 µl Guard1 (Cat. No. 635052)

B. Mammalian Cell Culture Supplies

- Tissue culture plates or flasks
- Cell culture medium
- Trypsin-EDTA (Trypsin; Sigma, Cat. No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; VWR, Cat. No. 82020-066; or Sigma, Cat. No. D8662)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. No. C6164 or C6039)
- Tet System Approved Fetal Bovine Serum (FBS; Clontech, Cat. Nos. 631101, 631105, 631106 & 631107)

C. Antibiotics for Clonal Selection

Prior to using antibiotics to select transduced cells, determine the optimal selection concentration for each cell type.

- Puromycin is available from Clontech (Cat. Nos. 631305 & 631306)
  —The recommended concentration range for viral systems is 1-2 µg/ml.

D. Additional Materials for Retro-X™ ProteoTuner Systems

- Retro-X Universal Packaging System (Cat. No. 631530)

NOTE: Please refer to the Retroviral Gene Transfer and Expression User Manual (PT3132-1, available at http://www.clontech.com/manuals) for a complete list of materials required.

- Retro-X qRT-PCR Titration Kit (Cat. No. 631453)

NOTE: Please refer to the Retro-X qRT-PCR Titration Kit User Manual (PT3952-1, available at http://www.clontech.com/manuals) for a complete list of materials required.

E. Additional Materials for Lenti-X™ ProteoTuner Systems

- Lenti-X HTX Packaging System (Cat. Nos. 631247 & 631249)

NOTE: Please refer to the Lenti-X Lentiviral Expression Systems User Manual (PT5135-1, available at http://www.clontech.com/manuals) for a complete list of materials required.

- Lenti-X 293T Cell Line (Cat. No. 632180)
- Tet System Approved Fetal Bovine Serum (Cat. Nos. 631101, 631105, 631106 & 631107)
- Lenti-X GoStix (Cat. Nos. 631241–631243), Lenti-X qRT-PCR Titration Kit (Cat. No. 631235), **or** Lenti-X™ p24 Rapid Titer Kit (Cat. No. 632200)

NOTE: Depending on the titration method you select, please refer to the Lenti-X GoStix Protocol (PT5123-2), the Lenti-X qRT-PCR Titration Kit User Manual (PT4006-1), or the Lenti-X p24 Rapid Titer Kit User Manual (PT5002-1; available at http://www.clontech.com/manuals) for a complete list of materials required.

III. ProteoTuner Assay Protocol Overview

A. Protein Stabilization

In order to stabilize your protein of interest, you can add the stabilizing ligand, Guard1, to one of two parallel cell cultures which were previously untreated with Guard1 (Figure 2, Panel A). The other culture will be continuously cultured in the absence of Guard1 as a negative control.

The added Guard1 will protect your DD_G-tagged protein of interest from proteasomal degradation, causing a dramatic increase in its level in the cell. Stabilization has been reported in as little as 60 minutes, but we recommend performing a time-course assay in order to determine the Guard1-based stabilization rate for your protein of interest, as well as testing different Guard1 concentrations (10 nM–1000 nM). At different time points, analyze the treated and control cells using your method of choice (e.g., western or phenotypic analysis), depending on your experimental goals.

B. Protein Destabilization

The default pathway of the ProteoTuner Systems is rapid destabilization and degradation of the tagged protein (Figure 1). In order to destabilize and degrade a tagged protein that has previously been stabilized, split the cells that were previously treated with Guard1 into two parallel cultures (Figure 2, Panel B). One culture will continue to be cultured in the presence of Guard1 as a positive control, and the second (experimental) culture will be cultured without Guard1.

In the absence of Guard1, the tagged protein of interest will be rapidly degraded. Degradation half lives of one to two hours have been reported (Iwamoto *et al.*, 2010), but we recommend performing a time-course assay in order to assess the rate of degradation of your protein of interest. At different time points, analyze the treated and control cells using your method of choice (e.g., western or phenotypic analysis), depending on your experimental goals.

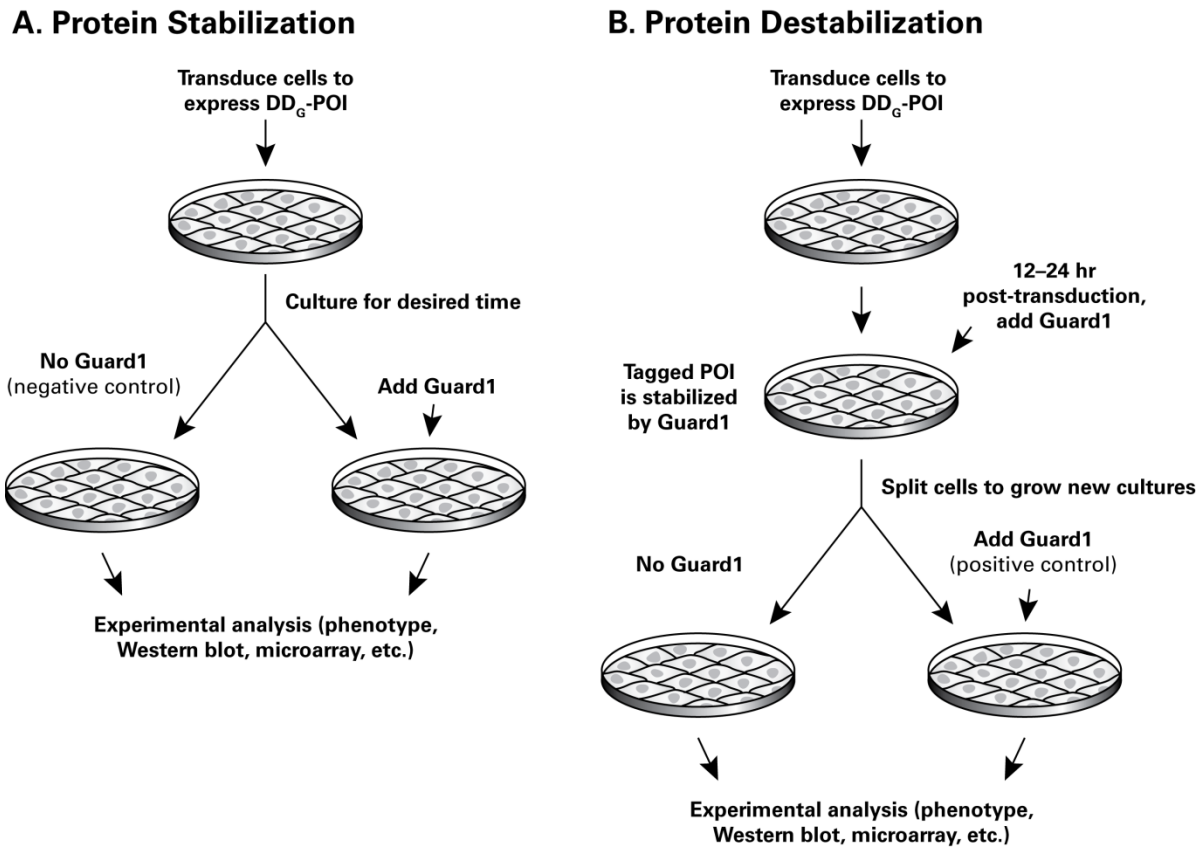

Figure 2. Overview of the ProteoTuner Guard System protein stabilization and destabilization protocols. Both protocols are based on Guard1's ability to reversibly stabilize DD_G-tagged fusion proteins (see Figure 1). Panel A. In order to observe the effects of stabilizing your protein of interest (POI), begin with cells cultured in medium that does not contain Guard1. Then add Guard1, and as your tagged-protein of interest is stabilized, perform your experimental analysis at defined time points in order to determine the protein's effects. Panel B. To observe the effects of the loss of your protein of interest, begin with cells cultured in medium that contains Guard1, and then split the cells into medium without Guard1 to destabilize your tagged-protein of interest. Then perform your experimental analysis at defined time points in order to determine the effects of the loss of your protein of interest.

IV. ProteoTuner System Protocols

A. Protocol: Propagating and Packaging ProteoTuner Viral Vectors

We offer both retroviral and lentiviral delivery of ProteoTuner technology. Each vector includes an antibiotic selection marker for transfection and transduction. There are two different DD_G tags available in both the retro- and lentiviral format: one is optimized for use as an N-terminal DD_G tag on a protein of interest, and the other for use as a C-terminal (C) DD_G tag.

Antibiotic selection: The **Retro-X ProteoTuner Guard Systems** and the **Lenti-X ProteoTuner Guard Systems** each include a puromycin resistance gene, which can be used to establish stable cell lines by selection with puromycin (not included; Cat. Nos. 631305 & 631306). For more information, please see Part II.C, Additional Materials Required.

1. To ensure that you have a renewable source of plasmid DNA, transform the vector provided with your system into a suitable *E. coli* host strain (e.g., Stellar™ Competent Cells, Cat. No. 636763–636767). See the vector CofA for further details.
2. To generate your desired construct, insert your gene of interest into the multiple cloning site (MCS) in-frame with the DD_G domain. When cloning into vectors that generate N-terminal tags, your gene of interest must contain a stop codon. When cloning into vectors that generate C-terminal tags, your gene of interest must contain a start codon and *lack* stop codons. We recommend using In-Fusion® Cloning Kits to perform your cloning.
3. Package your construct into retroviral or lentiviral particles:
   - **To generate retroviral particles** from your pRetro-X-PTuner2 construct, use the Retro-X Universal Packaging System (Cat. No. 631530) according to the Retroviral Gene Transfer and Expression User Manual (PT3132-1). You can use any method of choice to transfect your construct into the packaging cells.

     NOTE: The Retroviral Gene Transfer and Expression User Manual (PT3132-1) is available at http://www.clontech.com/manuals

   - **To generate lentiviral particles** from your pLVX-PTuner2 construct, co-transfect your construct and the Lenti-X HTX Packaging System (Cat. Nos. 631247 & 631249) into Lenti-X 293T Cells (Cat. No. 632180), according to the Lenti-X Lentiviral Expression Systems User Manual (PT5135-1). See the Certificate of Analysis (CofA) included with your pLVX-PTuner2 vector for further DNA propagation details.

     NOTE: The Lenti-X Lentiviral Expression Systems User Manual (PT5135-1) is available at http://www.clontech.com/manuals
4. Collect the viral supernatant 48–72 hours post-transfection. At this point you must determine your viral titer and infect your desired target cells.
   - **For retroviruses**, we recommend using the Retro-X qRT-PCR Titration Kit (Cat. No. 631453) to determine your viral titer.
   - **For lentiviruses**, we recommend using the Lenti-X qRT-PCR Titration Kit (Cat. No. 631235) to determine your viral titer.
5. Infect the target cells at a multiplicity of infection (MOI) of 0.5–5. A lower MOI guarantees lower background expression in the absence of the stabilizing ligand, which results in a greater "fold increase" of your tagged protein upon addition of the ligand.

B. Protocol: Optimizing Guard1 Concentration and Incubation Time

Before you begin, transduce your target cells with viral particles containing your construct (see Steps 3–5, above).

1. At 24–48 hours post-transduction, split the cells into at least two parallel cultures (i.e., one plate treated with Guard1 and one untreated negative control).

   Dilute the amount of Guard1 needed for your experiment:

   Guard1 is provided as a 0.5 mM stock solution, which should be diluted to its final concentration in warm (37°C) culture medium, and mixed well. We recommend trying a range of Guard1 concentrations from 10 to 1000 nM.

   You can dilute the Guard1 stock solution into medium that has already been used to culture the cells, or with fresh culture medium:

   - Collect the required amount of medium from your cell culture plate, place it into a sterile container, and add the appropriate amount of Guard1 to reach the desired final concentration. Mix well, and add the medium containing Guard1 back onto the plate.

     or

   - Warm the appropriate volume of fresh culture media needed for your experiment to ~37°C. Then add the appropriate volume of Guard1 stock solution, to obtain the final concentration of Guard1 to be used in the experiment.

     Example: Dilute Guard1 to 500 nM in10 ml of culture medium

     In a sterile conical tube, place:

     10 ml culture medium
     10 µl 0.5 mM Guard1
     10 ml 500 nM Guard1 (final conc.)

2. Optimize the concentration of Guard1:
   a. The final concentration of Guard1 used in the experiment may vary depending on the type of cells used, as well as your particular DD_G-tagged protein. Because the system is tunable, you can regulate or "tune" the amount of stabilized DD_G-tagged protein in your cells by adding either more or less of the stabilizing ligand Guard1 to the culture medium.
   b. We suggest testing various concentrations of Guard1: start by adding Guard1 to the culture medium to a final concentration of 1 µM, then test a range of concentrations above and below 1 µM to determine the optimal concentration range of Guard1 for your system. Because ProteoTuner systems are tunable, you'll be able to fine-tune the amount of your tagged protein by using a range of Guard1 concentrations.
3. Optimize the length of time the cells are incubated with Guard1.
   Your protein of interest may be detectable as early as one hour after the addition of the stabilizing ligand Guard1 (Iwamoto *et al.,* 2010). We recommend performing a time course assay in order to determine the optimal incubation time with Guard1.

C. Protocol: DD_G-Tagged Protein Stabilization/Accumulation

Before you begin, transduce your target cells with viral particles containing your construct (see Section IV.A, above).

i. Stabilizing DD_G-tagged proteins in adherent cells:

1. 24–48 post-transduction, split the cells into at least two parallel cultures (the number of plates depends on the number of samples you would like to collect).
2. Culture the cells (all plates) in medium without Guard1 until the cells attach to the plates.
   NOTE: Guard1 does not interfere with the attachment process. Therefore, Guard1 can also be added immediately after splitting if required for your experiment.
3. To fresh culture medium, add Guard1 to its optimal final concentration, as determined in Protocol B. We recommend using final concentrations of 10–1,000 nM Guard1.
4. Remove the medium from the culture plates, and replace it with warm medium containing or lacking Guard1. The Guard1 added to the experimental plate(s) will protect the DD_G-tagged protein from proteasomal degradation, causing a rapid increase in intracellular levels of the tagged protein.
6. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the Guard1-stabilized, DD_G-tagged protein.

ii. Stabilizing DD_G-tagged proteins in non-adherent cells:

1. 24–48 hours post-transduction, distribute the cell culture evenly into at least two tubes (the number of tubes depends on the number of samples you would like to collect), and centrifuge the tubes for 5 min at ≤ 1,000 rpm.
2. To fresh culture medium, add Guard1 to its optimal final concentration, as determined in Protocol B. We recommended using final concentrations of 10–1,000 nM Guard1.
3. Remove the supernatant from the pelleted cells, and resuspend the cells in warm medium containing or lacking Guard1 (prepared in step 2) as determined by your needs. The added Guard1 will protect your DD_G-tagged protein of interest from proteasomal degradation, causing a rapid increase in intracellular levels of the tagged protein.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the Guard1-stabilized, DD_G-tagged protein.

D. Protocol: DD_G-Tagged Protein Destabilization/Degradation

Before you begin, you will need to package your construct into viral particles, and use the particles to transduce your cells (see Section IV.A, above). Culture the transduced cells in medium containing Guard1 at the optimal concentration, determined in Protocol B, to stabilize the tagged protein.

i. Destabilizing DD_G-tagged proteins in adherent cells:

Method A

For fastest destabilization (requires splitting cells)

1. After adding Guard1 to the medium to stabilize the DD_G-tagged protein for the desired length of time, remove the medium containing Guard1.
2. Rinse the cells with warm Dulbecco's Phosphate Buffered Saline (tissue-culture-grade).
3. Detach the cells by your method of choice (trypsin, cell dissociation buffer, etc.) and split them into at least two new cell culture plates (the number of plates depends on the number of samples you would like to collect).

4. Culture the cells in one plate in medium containing Guard1 (positive control) and culture the cells in the other plate(s) in medium lacking Guard1. Growing the cells in the absence of Guard1 causes rapid degradation of the previously stabilized tagged protein.
5. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD_G-tagged protein.

Method B

For slower destabilization (no splitting required)

1. After using Guard1 to stabilize the DD_G-tagged protein for the desired length of time, remove the medium containing Guard1.
2. Wash the cells three times with warm medium lacking Guard1.
3. Culture the cells in medium lacking Guard1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD_G-tagged protein.

ii. Destabilizing DD_G-tagged proteins in non-adherent cells:

1. After adding Guard1 to the medium to stabilize the DD_G-tagged protein for the desired length of time, distribute the cell culture evenly into at least two tubes (the number of tubes depends on the number of samples you would like to collect).
2. Centrifuge the tubes for 5 minutes at ≤ 1,000 rpm then remove the supernatant.
3. Resuspend one cell pellet in medium containing Guard1at the appropriate concentration (positive control) and resuspend the remaining cell pellet(s) in medium without Guard1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD_G-tagged protein.

E. Protocol: Working with Stable Lines Expressing DD_G-Tagged Proteins

1. After establishing a stable cell line, you can culture your cells in either the absence or presence of Guard1, depending on your experimental needs.
2. If you grow your cells in the absence of Guard1, the destabilized DD_G-tagged protein will be present only at very low levels in your stable cell line. Guard1 can be added to rapidly increase the amount of your protein of interest as desired (Protocol D, above).
3. Maintenance in, or addition of Guard1 to a stable cell line will stabilize the DD_G-tagged protein, causing a rapid increase in intracellular levels of the protein (Protocol C).

V. References

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